Long-term hematologic reconstitution and clinical evaluation of autologous peripheral blood stem cell transplantation after cryopreservation of cells with 5% and 10% dimethylsulfoxide at –80°C in a mechanical freezer

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We report the long-term evaluation over 12 years of a simplified technique for stem-cell cryopreservation at –80°C without rate-controlled freezing and with 5% (n=251) or 10% (n=47) DMSO as the sole cryoprotectant. Platelet recovery was greater in the 5% DMSO group while long-term hematologic recovery did not differ. Factors influencing a faster hematologic recovery were infusion of more than $2.7 \times 10^6$ kg of CD34+ cells, 10% DMSO cryopreservation and G-CSF. We confirm that the procedure is feasible with a reduction in infusion-related toxicity from 60% using 5% DMSO. Differences in hematologic reconstitution were not clinically significant if a minimum of $1.5 \times 10^6$ kg CD34+ cells were infused.

Key words: autologous stem-cell transplantation, mechanical freezer, cryopreservation, DMSO

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ABSTRACT

Current protocols for hematopoietic stem cell (HSC) cryopreservation are usually based on the use of 10% dimethylsulfoxide (DMSO) as an intracellular cryoprotectant with or without hydroxethylstarch (HES) as an extracellular cryoprotectant. The toxic effects related to DMSO infusion are generally dose-related and while they are usually mild, they can become severe. HES is a relatively non-toxic drug but it is related with long-lasting pruritus and osmotic nephrotoxicity. Cryopreservation protocols usually involve rate-controlled freezing followed by the storage of the HSC in either the liquid or vapor phase of liquid nitrogen. These procedures are time consuming and require expensive computer-assisted devices.

Ten years ago we described a simplified –80°C HSC cryopreservation protocol that did not depend on rate-controlled freezing. This protocol involved storage in the same mechanical freezer in solutions containing 5% or 10% DMSO as the sole cryoprotectant without HES. Most studies seem to indicate that this simplified procedure is associated with a reduction in infusion toxicity and lower costs, with a similar hematopoietic reconstitution and clinical outcome to more standard protocols. However, there is little data regarding the long-term hematologic recovery and clinical course when such protocols are used.

Design and Methods

Patients

This study focuses on 298 consecutive patients who underwent autologous stem cell transplantation (ASCT) at the Hematology Services of the University Hospital Son Dureta and Policlinica Miramar in Palma de Mallorca, Spain. Patients were treated according to standard protocols. Grafts were cryopreserved without using a rate-controlled freezing protocol and they were stored in the same mechanical freezer in 10% (from July 1993 to September 1995; n=47) and 5% (from March 1993 to April 2005; n=251) DMSO without HES after informed consent was obtained. The main characteristics of the two patient groups and transplant-related procedures are presented in Table 1.
Mobilization, collection and cryopreservation of HSC

In most cases (87%), the mobilization regimen used involved daily subcutaneous administration of G-CSF (filgrastim) at 5 μg/kg of body weight. In the remaining patients, a combination of chemotherapy and G-CSF was used. The HSC were collected with a cell separator (CS-3000 Plus, Fenwal Laboratories, Deerfield, IL, USA). The cryoprotectant solution used in this study contained autologous plasma, 10% ACD and 20% or 10% DMSO as the sole cryoprotectant. This solution was mixed with an equal volume of the cells obtained through apheresis at 4°C. The final cell concentration was adjusted to <100 × 10^9/L with a final concentration of DMSO of 10% or 5%. After mixing, the HSC were distributed into plastic bags (Cryocyte, Fenwal) and introduced into a rack in a –80°C mechanical freezer (Revco Scientific, Inc., Asheville, or Heraeus, Hanau, Germany).

Biological studies

Cell counts were obtained using an automated cell counter Advia 120 (Bayer Diagnostics). The viability of mononuclear cells was studied by trypan blue-dye exclusion. We performed flow cytometric analysis, using anti-CD33 fluorescein isothiocyanate and anti-CD34 phycoerythrin on a flow cytometer (FACScan, Becton Dickinson). The CFU-GM assay was performed with the stem cell CFU kit (GIBCO BRL, Grand Island, NY, USA).

Discussion and Results

Post-thaw analysis of hematopoietic infused grafts

No significant differences were observed in median storage times, post-thaw nucleated cell viability, or number of infused CD34+ or CFU-GM cells between the cells cryoprotected in 5% and 10% DMSO (Table 1). Indeed, cryopreservation time did not influence hematopoietic cell viability with either 5% and 10% DMSO (R=0.092; p=0.37), considering that the HSC were cryopreserved at –80°C in a mechanical freezer for the most part for less than six months.

Toxicity of the infused cells

The infusion-related toxicity reached 19.1% in the 10% DMSO group. This is significantly higher than the 6.8% observed in the 5% DMSO group (p=0.006) (Table 2). Furthermore, the mild infusion-related toxicity of 19.1% in the 10% DMSO group was more than four-fold that in the 5% DMSO group (4.4%), as shown by chills, skin rash and abdominal pain (p=0.001).

Engraftment and early hematopoietic recovery

Early hematopoietic recovery is shown on Table 1. An univariate analysis with the Mann Whitney U test was carried out to identify the factors influencing hematopoietic recovery: Gender, diagnosis, CD34+ cells infused, cryopreservation DMSO concentration type, cell viability and posttransplant G-CSF were all significant for days to ANC >0.5 × 10^9/L. Diagnosis, CD34+ cells infused, CFU-GM cells infused and cryopreservation DMSO concentration type were all significant for days to platelet recovery to >20 × 10^9/L and together with cell viability were also significant days to recovery to >50 × 10^9/L. In the multivariate
regression analysis three main factors were associated with the recovery of ANC to 0.5×10^9/L: infusion of >2.7×10^6/Kg CD34+ cells (p<0.001); postransplant G-CSF administration (p<0.001); and cryopreservation with 10% DMSO (p=0.022). However, only the number of CD34+ cells significantly influenced the more rapid recovery of platelets in the multivariate setting (p<0.005). Nevertheless, we maintained the DMSO concentration in the regression models because it modulates the ability of any given dose of CD34+ infused cells to reduce recovery time (Figure 1).

Long-term hematopoietic recovery

There was no difference in the long-term hematologic parameters at 6, 12 and 24 months between the two groups of patients transplanted with hematopoietic cells cryopreserved with 5% or 10% DMSO. Engraftment was safe and sustained in the vast majority of the patients evaluated (99.2%). Two patients (0.8%) in the 5% DMSO cryopreservation group experienced engraftment problems: a graft failure in 1 case of chronic lymphoid leukemia and a delayed engraftment with blood transfusion dependence beyond 24 months in 1 case of lymphoblastic lymphoma. The first patient died 40 days after transplantation due to an infection related to the graft failure. By contrast, the second patient finally reached normal peripheral blood counts after more than three years after transplantation.

Outcome and long-term follow-up data

Median follow-up for alive patients was 127 (50-139) months for those receiving 10% DMSO and 57 (2-128) months for the 5% DMSO group. In this period, 148 (50%) patients were still alive, 25 (55%) and 123 (49%) in the 10% and 5% DMSO groups respectively. This study presents the results of the long-term follow-up after using a simplified method of HSC cryopreservation at –80°C that was first described by our group ten years ago. To our knowledge, this is the largest and most comprehensive study using such a method, focusing on toxicity, outcome and both the short-term and long-term hematologic recovery in 298 patients over a 12 year follow-up. In agreement with previous studies,10-12 our multivariate analysis confirmed that the CD34+ cell dose is an important prognostic factor influencing both short-term neutrophil and platelet engraftment. G-CSF administration also promoted a faster recovery of neutrophils. Interestingly, the inclusion of DMSO concentration in the regression model confirmed the influence of the type of cryopreservation in neutrophil and platelet recovery. In fact, when 5% DMSO was used for cryopreservation, a slower hematologic recovery was observed compared with the 10% DMSO group, despite having received a median higher amount of CD34+ cells. This could suggest that 10% DMSO concentration improves CD34+ cells protection or conservation allowing the same dose of CD34+ cells in the 10% DMSO group to induce a faster hematologic recovery than a similar dose in the 5% DMSO group (Figure 1). However, these differences did not seem to be clinically relevant. The short-term hematologic recovery was also slightly slower in the patients subjected to cryoprotection with 5% DMSO compared with the 10% DMSO group. Previous reports found a similar slightly slower hematopoietic recovery when comparing stem cell cryopreservation with DMSO with or without HES,13 with or without controlled-rate freezing14 as well as with different concentrations of DMSO.15 More importantly, two incidences of poor or incomplete engraftments occurred when

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**Table 2. Procedure-related toxicity.**

<table>
<thead>
<tr>
<th></th>
<th>Group 5% DMSO</th>
<th>Group 10% DMSO</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>251</td>
<td>47</td>
<td>0.006</td>
</tr>
<tr>
<td>Total patients with significant IRT (%)</td>
<td>17 (6.8%)</td>
<td>9 (19.1%)</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>11 (4.4%)</td>
<td>6 (12.8%)</td>
<td></td>
</tr>
<tr>
<td>Tachycardia</td>
<td>3 (1.2%)</td>
<td>2 (4.2%)</td>
<td></td>
</tr>
<tr>
<td>Bradycardia</td>
<td>2 (0.8%)</td>
<td>2 (4.2%)</td>
<td></td>
</tr>
<tr>
<td>Chest tightness</td>
<td>1 (0.4%)</td>
<td>1 (2.1%)</td>
<td></td>
</tr>
<tr>
<td>Total patients with mild IRT (%)</td>
<td>11 (4.4%)</td>
<td>8 (19.1%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Chills</td>
<td>6 (2.4%)</td>
<td>4 (8.5%)</td>
<td></td>
</tr>
<tr>
<td>Skin rash</td>
<td>4 (1.6%)</td>
<td>1 (2.1%)</td>
<td></td>
</tr>
<tr>
<td>Bone/abdominal pain</td>
<td>1 (0.4%)</td>
<td>3 (6.4%)</td>
<td></td>
</tr>
<tr>
<td>Transplantation-related mortality</td>
<td>6 (2.4%)</td>
<td>1 (2.1%)</td>
<td>NS</td>
</tr>
<tr>
<td>Infection</td>
<td>5 (2.0%)</td>
<td>1 (2.1%)</td>
<td></td>
</tr>
<tr>
<td>Graft failure</td>
<td>1 (0.4%)</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Second neoplasia</td>
<td>4 (1.6%)</td>
<td>2 (4.2%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

*A patient may display one or more adverse effects.*

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Figure 1. Linear regression models of hematological recovery according to the concentration of DMSO used. The 10% DMSO group needs smaller amounts of CD34+ infused cells to achieve a faster speed of engraftment of neutrophils and platelets illustrated by a faster gradient.
HSC was preserved in 5% DMSO. In both cases the patients received relatively low doses of CD34+ cells (0.6 and 1.2×10^6/Kg respectively). However, in the group of 10% DMSO, a significantly lower number of CD34+ cells were infused without introducing any problems of engraftment problems. Similarly, no engraftment problems were observed when HSC was cryopreserved with 5% DMSO and the patients were infused with more than 1.5×10^6/Kg CD34+ cells. At the same time, an equivalent long-term hematologic recovery was experienced between the two groups studied, showing that the slight delay in the initial recovery is transient and may not have any clinical relevance. Long-term observations in this study confirm previous data regarding the feasibility of cryopreserving stem cells in diminished DMSO concentrations; as low as 2.2% for stem cells cryopreserved in liquid nitrogen or as low as 5% for stem cells frozen and stored at –80°C.13–16 This study also confirms that the elimination of HES as an extracellular cryoprotectant can be carried out within the cryopreservation time scale of less than 6 months without affecting long-term hematologic recovery. However, we recommend caution when considering longer periods of cryopreservation.9

Another important point to be considered is that the infusion of cells cryopreserved in the lowest concentration of DMSO (5%) without HES reduced both the significant and the mild infusion-related toxicity by more than 60%. This reduced toxicity appears to be the most significant advantage of cryopreservation in 5% DMSO. Furthermore, this data agrees with a recently published report on the toxicity of DMSO based on the information collected in a questionnaire in 97 EBMT centers.15 At the same time, storage of HSC in a mechanical freezer is cheaper and simpler, and avoids the possible contamination associated with the use of nitrogen tanks.20

Authors’ Contributions
AGa, AGú, AS, MC, JBa, JBe were responsible for conception and design, analysis and interpretation of data and drafting the article; MM, JJ, NM, MD, AN, LY, PG, JBA and JM were responsible for acquisition of the data and drafting the article. Finally, Joan Besalduch was responsible of the final approval of the version to be published.

Conflict of Interest
The author reported no potential conflicts of interest.

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